Expression of DDR1 and DDR2 in Peripheral Blood

Undergraduate Honors Thesis

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Abstract

Upon injury to the vessel wall, interactions between platelets and the subendothelial collagen play a critical role in initiating the clotting cascade. The modulation of platelet-collagen adhesion by non-platelet derived receptors present in the peripheral blood is not well-understood. Discoidin domain receptors (DDRs) are a unique family of collagen-binding receptors that are activated by their ligand collagen, and play an important role in collagen remodeling. This study aims to determine expression of DDRs in leukocyte and in platelets in the human peripheral blood with the long term goal of identifying the role of DDRs in modulating platelet-collagen interactions.

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Introduction

Platelet-collagen interactions play crucial roles in clinically relevant scenarios such as stroke, atherosclerosis, and myocardial infarction. Arterial thrombosis, a leading cause of myocardial infarction, is initiated when intimal collagen is exposed to the flowing peripheral blood. Platelets begin the coagulation cascade when they adhere to collagen via the GPVI receptor and to collagen bound vWF via the GP1b receptor present on the platelet surface. Besides GPVI and GP1b, the integrins $\alpha_2\beta_1$ present on the platelet surface are also known to mediate platelet-collagen interactions. In recent years several new collagen receptors have been identified. However, their expression in peripheral blood cell-types has not been well-characterized. This is especially important as non-platelet derived collagen receptors may also play a role in modulating platelet-collagen interactions.

Receptor tyrosine kinases (RTKs) are transmembrane receptors which play crucial roles in cell-signaling[1]. The discoidin domain receptors DDR1 and DDR2 are a family of RTKs which consist of an extracellular domain (ECD), transmembrane domain, and intracellular kinase domain[1, 6] (Figure 1). DDR1 and DDR2 are differentiated from other RTKs in that their ECDs bind to and are activated by triple helical collagens[1]. Interactions between DDRs and collagen have been shown to play roles in the migration, proliferation, differentiation, and survival of multiple mammalian cell types [1, 2, 3]. Dysregulation of the DDRs are implicated in the

pathogenisis of multiple disease states, including atherosclerosis[2, 3], cancer[4, 5], and myocardial infarction[1, 5].

The current study aims to evaluate the expression of DDR1 and DDR2 in human peripheral blood via the following specific aims.

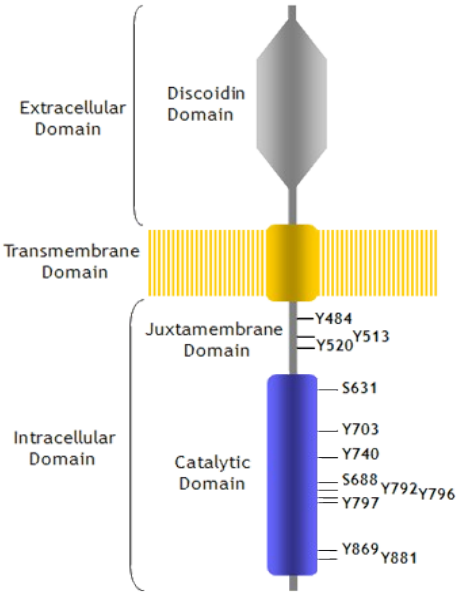

Figure 1: DDR structure. The exctracellular discoidin domain is activated when bound to collagen, leading to autophosphorylation of intracellular tyrosine residues[6].

Specific Aim: Determine expression of DDR1 and DDR2 in leukocytes and platelets. mRNA from human platelets and leukocytes was used to analyze for DDR expression using PCR based approach.

Methods

Blood Collection and Platelet Preparation

8 mL of whole blood from available healthy donors was drawn into either sodium citrate or EDTA containing tubes via our approved IRB protocol. Blood samples were centrifuged at 200 x g for 15 minutes, resulting in a translucent layer containing platelet-rich-plasma (PRP), a buffy coat (BC) containing leukocytes, and an opaque red layer containing red blood cells (RBC). The PRP layer was seperated from the BC layer using wide bore pipette tips and the RBC layer was discarded. PRP and BC were centrifuged at 5000 x g for 5 minutes to yield solid cell pellets consisting primarily of platelet or leukocytes respectively. The cell pellets were rinsed with room temperature PBS, and centifuged again at 5000 x g for 5 minutes to regain the cell pellet. Cells were lysed in 250 uL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and stored at -20 ^{o}C.

mRNA Precipitation

mRNA precipitation followed a modified version of the manufacturer's protocol included with the TRIzol Reagent. Cell homogenates were incubated for 5 minutes at room temperature to allow nucleoprotein complexes to dissociate. 50 uL of choloroform was added, and homogenates were vortexed and incubated another 2 minutes at room temperature. The samples were then centrifuged at 12000 x g for 15 minutes at 4 ^{o}C, separating the mixture into a lower red phase and, and a colorless upper phase containing mRNA. The colorless phase was carefully

transferred to a new tube, while the rest was discarded. The mRNA was precipitated by adding at least 125 uL of isopropyl, freezing the mixture at -80 ºC for at least an hour, and centrifuging at 12000 xg for 10 minutes at 4 ºC. The mRNA precipitate formed as a nearly invisible pellet on the bottom of the tube. After removing the supernatant, the mRNA pellet was washed twice by adding 0.5 mL of 75% ethanol, vortexing to break up the pellet, and re-precipitation by centrifuging at 7500 x g for 5 minutes at 4 ºC. The ethanol was removed from the tube, and the mRNA was allowed to air dry for about ten minutes, or until the mRNA pellet was translucent. We dissolved the mRNA in 20 uL of RNase-free water by repeatedly passing the pellet through a pipette tip and incubating for 10 minutes at 60 ºC. The 260/280 ratio of the mRNA was measured using a Beckman Coulter 730 Spectrophotometer (Beckman Coulter, Inc., Pasadena, CA, USA). Only samples with a 260/280 ratio above 1.6 were used. mRNA purity was also assessed by running PCR using DDR1 primers (Table 1), which shows an additional 359 bp band due to genomic DNA contamination (Figure 2). The mRNA samples were stored at -20 ºC.

Figure 2 : Representative gel showing detection of DNA contamination. Bands at 359 bp are PCR products of DNA, while lighter bands at 100 bp are the correct mRNA products.

First-Strand cDNA Synthesis

cDNA synthesis from isolated mRNA followed the protocol included with SuperScript II RT (Invitrogen). 500 ng of isolated mRNA was mixed with 1 uL Oligo(dT) (Invitrogen) , 1 uL dNTP Mix (Initrogen), and RNase-free water to a final reaction volume of 12 uL. The mixture was heated to 65 ^{o}C for 5 minutes and chilled on ice. Thereafter 4 uL First-Strand Buffer (Invitrogen) and 2 uL 0.1M DTT (Invitrogen) were added, mixed by gently passing through pipette tip, and incubated at 42 ^{o}C for 2 minutes. 1 uL of SuperScript II RT (Invitrogen) and Rnase-free water were added to a final volume of 20 uL, and the mixture was allowed to sit at room temperature for 10 minutes. The reaction was completed by incubation at 42 ^{o}C for 50 min, followed by incubation at 70 ^{o}C for 15 minutes. cDNA samples were stored at -20 ^{o}C.

PCR

PCR reactions were prepared using 25-100 ng first-strand cDNA, 5 uL PCR Buffer in $MgCl^{2+}$ (Hoffman-La Roche, Basel, Switzerland) , 1 uL dNTP Mix, 5 uL forward primer, 5 uL reverse primer, and 0.4 uL Taq DNA Polymerase (Hoffan-La Roche). The mixtures were heated to 95 ^{o}C for 4 minutes, then went through 40 cycles of denaturation at 95 ^{o}C for 1 minute, annealing at primer-specific annealing temperatures (Table 1) for 1 minute, elongation at 72 ^{o}C for 2 minutes, and a final elongation at 72 ^{o}C for 7 minutes. PCR products were stored at -20 ^{o}C.

Table 1: List of primer sequences, molecular weights of mRNA PCR products, and primer melting temperatures. DDR primers taken from Ruiz et al.[7], GP1ba and HLADQb primers taken from Paul et al.[8]

	DDR1	DDR2	GP1ba	HLADQb
Forward Primer	GCG TCT GTC TGC GGG TAG AG	TGT TCC TGC TGC TGC CTA TCT T	GGT GCG TGC CAC AAG GAC TGT	GTC TCA ATT ATG TCT TGG AA
Reverse Primer (reverse complement)	AGA CAA TGT ATT TAT CTG AGG CCG T	ATC CAG CTA TAT GCC GCT ATC CT	CGT CCC GGA GCC CGC CCC AAA	AGC AAG ATG CTG AGT GGC
mRNA MW (bp)	100	68	282	300
Melting Temp (Degrees Celsius)	69(F) 66.5(R)	68.8(F) 67.6(R)	73.7(F) 77.8(R)	58.3(F) 65.6(R)

Gel Electrophoresis

PCR products were separated based on molecular weight using standard gel electrophoresis protocols. PCR products were loaded into 1.5% agarose gels, allowed to run for 45 minutes at 100 V, and imaged in UV light.

Results

Peripheral blood samples obtained from n=3 healthy subjects was utilized to obtain mRNA from the leukocyte fraction and the platelet fraction. Two platelet mRNA samples (PL1: from top 1 ml of PRP) and PL2: second 1 ml of PRP) and one leukocyte mRNA sample (WBC) were isolated from fractions, and converted to cDNA. All three samples demonstrated 260/280 ratios above 1.6, and tested negative for DNA contamination using PCR as described in the methods section. PCR was run using 100ng template cDNA from all three samples.

Figure 3 shows results obtained using PCR. . The WBC sample tested positive for HLA-DQb, a leukocyte specific gene while PL1 and PL2 both tested negative. All three samples displayed the bands corresponding to GP1ba, a platelet specific marker. This was expected, as our buffy coat layer contains both platelets and leukocytes, while the PL samples contain only platelets.

We then tested for expression of DDR1 and DDR2 in leukocyte and platelet samples. Plasmids containing DDR1 (DDR1-P) and DDR2 (DDR2-P) were used as both positive and negative controls. WBC samples showed bands for both DDR1 and DDR2. However neither PL1 or PL2 displayed bands for DDR1 or DDR2.

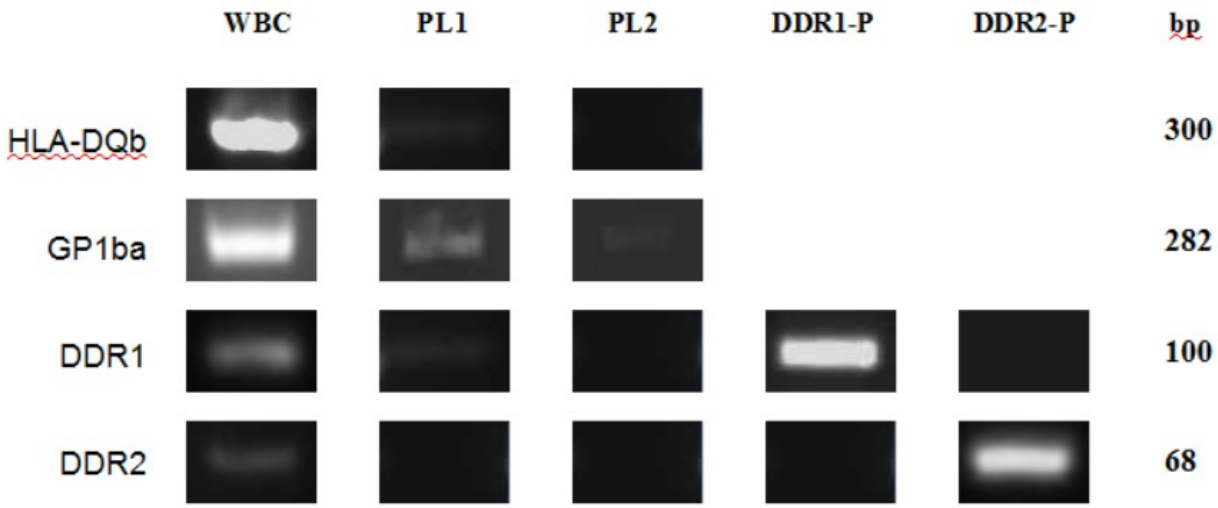

Figure 3 : PCR products from mRNA samples WBC, PL1, and PL2 as well as control plasmids DDR1-P and DDR2-P were loaded into 1.5% agarose gels and run at 100 V for 45 minutes. Leukocyte expression of DDR1 and DDR2 was demonstrated, while platelets did not express either DDR.

Discussion and Conclusion

DDR1 and DDR2 are expressed in leukocytes. These results confirm earlier Western Blot analysis performed in our lab, as well as in the literature[9]. While the bands in Figure 3 are relatively weak, we believe they are prominent enough to confirm expression, and future work aim at strengthening the bands by optimizing the PCR reaction. Expression of DDR1 and DDR2 in leukocytes support previous studies indicating DDR mediates leukocyte-collagen interactions during inflammation[2].

Platelets do not express DDR1 or DDR2. Relatively little research has focused on expression of DDR1 and DDR2 in peripheral blood. A study by Abonnante et al.[10] used Western Blot analysis to show that platelets express DDR1, which disagrees with our results. In this earlier study, leukocyte contamination of platelet samples and/or non-specific binding of DDR1 antibody has not been well-characterized. Our use of both positive and negative plasmid controls in determining DDR1 and DDR2 expression in platelets creates a more stringent analyis, indicating that platelets do not express either DDR.

Overexposing the gel revealed very faint bands for PL1 using both HLA-DQb and DDR1. The HLA-DQb band indicates possible leukocyte contamination, which could in turn be responsible for the DDR1 band. Future studies should seek to ensure complete purity of platelet samples,

possibly by utilizing methods described by Paul et al.[8]

Understanding the expression of discoidin domain receptors in different blood fractions will allow us to further understand platelet-collagen interactions in the whole blood.

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